Incidence of two virulence factors (aerobactin and mucoid phenotype) among 190 clinical isolates of *Klebsiella pneumoniae* producing extended-spectrum β-lactamase

V. Vernet a, C. Madoulet a,b, C. Chippaux a and A. Philippon c

a Faculté de Médecine, Laboratoire de Bactériologie-Virologie, Reims, France, b Faculté de Pharmacie, Laboratoire de Biochimie, Reims, France, and c Faculté de Médecine Lariboisière St. Louis, Laboratoire de Bactériologie-Virologie, Paris, France

Received 13 April 1992
Revision received and accepted 3 June 1992

Key words: Extended-spectrum β-lactamases; Aerobactin; Mucoid phenotype; Virulence factors; *Klebsiella pneumoniae*

1. SUMMARY

Because outbreaks of multiple-resistant *Klebsiella pneumoniae* isolates producing extended-spectrum β-lactamases were recently observed in French hospitals, the presence of virulence factors was examined for (i) phenotype by bioassay for aerobactin production and by culture for the mucoid phenotype, and (ii) genotype using intragenic probes of respectively 2-kb *Bgl* II and 235-bp *Bam*H I-*Bgl* II fragments and dot-blotting among 190 unreplicated *K. pneumoniae* clinical isolates issued from 25 French hospitals and producing different types of extended-spectrum β-lactamases (TEM-related enzymes: TEM-3, TEM-4, CAZ-1, CAZ-2, TEM-8, or SHV-related enzymes: SHV-2, SHV-3, SHV-4). Only 3.7% and 7% of *K. pneumoniae* isolates produced aerobactin and mucoid phenotypes respectively, unrelated to type of β-lactamase. Only 2% had both factors. No discordance was reported according to the detection method tested. The low prevalence of such virulence factors seems to indicate they were not involved in dissemination of nosocomial *K. pneumoniae* isolates producing an extended-spectrum β-lactamase.

2. INTRODUCTION

*Klebsiella pneumoniae* causes suppurative and sometimes life-threatening infections. It is responsible for extensive outbreaks of infection which mainly involve compromised patients. Several *K. pneumoniae* virulence factors have been clearly individualized including the polysaccharide capsules such as K1 and K2 which protect the organism against ingestion and killing by professional phagocytes [1,2], lipopolysaccharide which inhibits the bactericidal effect of serum complement [2,3], and adherence-mediated pili
Two plasmid-encoded factors, aerobactin and mucoid phenotype, have recently been described as being required for the high pathogenicity of *K. pneumoniae* in mice [5,6]. In *Escherichia coli* isolated from humans, the presence of virulence factors, for example plasmid-encoded aerobactin, is often associated with antibiotic resistance, and such strains expressing virulence factors are often isolated from compromised patients [7,8].

*K. pneumoniae* isolates with multiple antibiotic resistance have been shown to be widespread in French hospitals ([9,10] and for review see [11]). Many of them produce plasmid-encoded extended-spectrum β-lactamases and are also resistant to aminoglycosides (tobramycin, amikacin) [12]. These extended-spectrum β-lactamases inactivate a wide range of β-lactams including third generation cephalosporins [12], and are now found on all continents [13,16].

There has been no study of the association, if any, of the production of extended-spectrum β-lactamases with that of virulence factors, either in *K. pneumoniae* or *E. coli*. We therefore determined whether or not the virulence factors, aerobactin production and mucoid phenotype, are associated with the production of extended-spectrum β-lactamases. A group of 190 clinical *K. pneumoniae* isolates from 25 French hospitals isolated between 1984 and 1988 was investigated.

### 3. MATERIALS AND METHODS

#### 3.1. Bacterial isolates and plasmids

190 clinical isolates were used in this study. They were recovered from patients hospitalized between 1984 and 1988, mainly in intensive care units, in 25 French hospitals. These isolates, responsible for nosocomial infections, were isolated from blood (30%), urine (60%) and other samples (wound, stools, sputum, etc.). Laboratory strains are listed in Table 1 and were provided by Dr. Nassif (Unité de Pathogénie Microbienne, Institut Pasteur, France).

#### 3.2. β-Lactamase assays and characterization

Crude sonicated extracts were prepared from overnight cultures at 37°C in trypticase soy broth

(Difco, France). Isoelectric focusing was performed on polyacrylamide gels as elsewhere described [9,13,19] and β-lactamase activity was located on the gels both with a 100-ml iodine-starch agar gel containing 20 mg of ceftriaxone and with the classic nitrocefin method. Complementary β-lactamase identification tests (substrate profile, inhibition profile, DNA probes) were performed for some strains [13,14,19,20].

#### 3.3. Phenotypic detection of aerobactin production

The production of aerobactin was demonstrated by a cross-feeding bioassay using *E. coli* strain LG1522 [18]. The clinical isolates to be tested for aerobactin production were grown overnight in M9 broth [21] containing the iron chelator α-α' dipipyridyl (200 μM) (Sigma, St. Louis, MO). Strains were spotted onto hardened dipipyridyl-minimal agar plates. After 18 h at 37°C satellite growth of the indicator strain LG1522 around the disks indicated aerobactin production [5].
3.4. Phenotypic detection of mucoid phenotype
Each isolate was plated on trypticase soy agar (Difco, France) and the plates incubated for 18 h at 37°C as previously described [6]. The mucoid aspect of the strains was scored.

3.5. Genotypic detection of the aerobactin and mucoid phenotypes

3.5.1. Preparation of probes. The aerobactin probe was prepared from pKP4 DNA [5]. Plasmid DNA was digested with BglII restriction endonuclease (Bethesda Research Laboratories, Gaithersburg, MD) and electrophoresed on a 2% agarose gel. The 2-kb BglII fragment was excised from the gel, electroeluted, cleaned by isopropanol precipitation, and labeled by random priming [22] with $^{32}$P (Amersham, Buckinghamshire, UK). The mucoid phenotype probe was prepared from pKP228 [6]: plasmid DNA was digested with BamHI and BglII restriction endonucleases, electrophoresed in a 5% polyacrylamide gel. The probe, a 235-bp BamHI-BglII fragment, was isolated and prepared as above.

3.5.2. Plasmid DNA extraction and hybridization. Plasmid DNA of K. pneumoniae strains was extracted by the method of Kado and Liu [23], and spotted onto nitrocellulose filters (Nytran membranes, Schleicher & Schuell, Dassel, FRG) [24]. The probes ($10^7$ dpm/ml) were hybridized to the membranes as previously described [25] under stringent conditions. The filters were exposed to X-Omatic Kodak (Eastman Kodak, Rochester, NY) for 72 h at −70°C [25].

4. RESULTS

4.1. Distribution of extended-spectrum $\beta$-lactamases
The distribution of extended-spectrum $\beta$-lactamases was: 75 TEM-3, 8 TEM-4, 2 TEM-5 (CAZ-1), 3 CAZ-2, 14 SHV-2, 22 SHV-3, 65 SHV-4 (Table 2) and 1 TEM-8 (not shown). The isolate producing 1 TEM-8 did not produce virulence factors (not shown).

4.2. Incidence of aerobactin and mucoid phenotype
Seven isolates (3.7%) produced aerobactin (Table 2). The extended-spectrum $\beta$-lactamases produced by these isolates were: TEM-3 (3), SHV-2 (2), SHV-4 (2).

Thirteen isolates (7%) had a mucoid aspect on trypticase soya agar. These isolates produced TEM-3 (7), TEM-4 (1), SHV-2 (3), SHV-3 or SHV-4 (1). Four strains (2%) both produced aerobactin and had a mucoid phenotype. They produced TEM-3 (2 isolates) and SHV-2 (2 isolates) (Table 2).

All the isolates positive for these virulence factors (Table 2) hybridized with the two intragenic probes and we found no hybridizing strain isolate which did not have the corresponding phenotype.

4.3. Distribution of virulence factors among K. pneumoniae isolates, from 25 French hospitals
As shown in Table 2, no correlation was detected between K. pneumoniae isolates producing aerobactin or mucoid phenotype and the geographic region in which the isolates were found. In the previously described hospital outbreaks [9,13,14], virulence factors were not investigated.

<table>
<thead>
<tr>
<th>$\beta$-Lactamases</th>
<th>Number of isolates</th>
<th>Aerobactin production (A)</th>
<th>Mucoid phenotype (M)</th>
<th>A + M production</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-3</td>
<td>75</td>
<td>3 (2)</td>
<td>7 (4)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>(CTX-1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM-4</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TEM-5</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>(CAZ-1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAZ-2</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>SHV-2</td>
<td>14</td>
<td>3 (3)</td>
<td>4 (4)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>SHV-3</td>
<td>22</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>SHV-4</td>
<td>(CAZ-5)</td>
<td>65</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total isolates (%)</td>
<td>190</td>
<td>7 (3.7)</td>
<td>15 (7)</td>
<td>4 (2)</td>
</tr>
</tbody>
</table>

a ( ): number of hospitals.
5. DISCUSSION

Extended-spectrum β-lactamases are a worldwide problem [3]. Outbreaks of nosocomial infections due to Enterobacteriaceae producing these enzymes are mainly caused by K. pneumoniae and usually in intensive care units [4–7]; this species ranks second to Escherichia coli as the most common cause of Gram-negative infection [26]. We screened 190 extended-spectrum β-lactamase-producing K. pneumoniae isolates, involved in nosocomial infections and in outbreaks, for the virulence factors’ aerobactin production and mucoid phenotype [13,14]. The proportion of the isolates displaying these virulence factors was low: 3.7% for aerobactin, 7% for mucoid phenotype, and only 2% for both. The correlation between biochemical and genetic methods seems similar to that previously described [27]. But our results contrast with the earlier studies of aerobactin production, in which 15% or more of the K. pneumoniae isolates were aerobactin producers [28,29]. The difference could be due to the fact that only 30% of our isolates were recovered from blood culture. Moreover, the plasmid expressing this phenotype could have been lost by the method we used for storing the strains (on agar at room temperature).

When we analyzed the virulence factor positive isolates for linkage with type of extended-spectrum β-lactamase produced (TEM-related enzymes (TEM-3/CTX-1, TEM-5/CAZ-1), or SHV-type (SHV-2, SHV-3 or SHV-4CAZ-5), no linkage was found. Finally, virulent isolates were not issued from the same hospital, a fortiori from the same unit.

Extended-spectrum β-lactamases are an emerging problem which is due to the liberal use of antibiotics. However, the factors causing outbreaks of K. pneumoniae producing such enzymes remain obscure. This study suggests that aerobactin production and mucoid phenotype are not directly involved in the worldwide emergence and epidemiological prevalence of K. pneumoniae producing extended-spectrum β-lactamase. Other studies [9,10] suggest that the presence of capsule seems to contribute significantly to virulence in this bacterial species.

ACKNOWLEDGEMENTS

We acknowledge N. Tandart for secretarial assistance. We thank X. Nassif and P.J. Sansonetti for providing strains.

REFERENCES


